2010 Project Abstract
For the Period Ending June 30, 2013

PROJECT TITLE:	Ecological Impacts of Effluent in Surface Waters and Fish
PROJECT MANAGER:	Paige J. Novak, Ph.D., P.E.
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FUNDING SOURCE:	Minnesota Environment and Natural Resources Trust Fund
LEGAL CITATION:	M.L. 2010, Chp. 362, Sec. 2, Subd. 5c

APPROPRIATION AMOUNT: $340,000

Overall Project Outcome and Results

Phytoestrogens are plant-based compounds that mimic estrogen and can interfere with normal biological development. Research shows that phytoestrogens are discharged into surface water from wastewater treatment plants and certain industries. The biological effects of these compounds have not been well studied, although it is known that they can feminize male fish. Almost nothing is known about their environmental fate. When these compounds enter rivers and streams, it is likely that they will be degraded and therefore may have a lessened impact on biota, but this needs to be confirmed

In this project, the persistence of two common phytoestrogens (genistein and daidzein) was studied. Fathead minnow exposure experiments at realistic environmental concentrations were also performed. Experiments demonstrated that genistein and daidzein reacted with sunlight. These two compounds also biodegraded rapidly in natural water samples; the rate of degradation depended on phytoestrogen concentration, water/incubation temperature, and the source of the water. Sorption experiments showed that phytoestrogens sorb to sediment, but this is not likely to be an important loss mechanism. Adult fathead minnow exposure experiments showed that only subtle effects on anatomy, physiology and behavior of fathead minnows occurred as a result of exposure to phytoestrogens singly or in mixtures. The one exception to this was the fact that adult fathead minnows produced significantly more eggs when exposed to daidzein. Larval minnow exposures showed that exposure to genistein, formononetin (another common phytoestrogen), and a mixture of phytoestrogens had a negative impact on larval survival. Adult and larval exposures to microbiologically degraded phytoestrogens showed negative impacts on adult egg production. This research indicates that genistein, daidzein, and formononetin are unlikely to cause widespread ecological harm themselves in the absence of other stressors; nevertheless, caution should be exercised with respect to high concentration effluents due to the potentially anti-estrogenic effects of phytoestrogen degradates.

Project Results Use and Dissemination

Results have been disseminated at several conferences. In addition, one manuscript has been published, two additional manuscripts have been submitted, and a fourth is being revised and will be submitted for publication in August or September, 2013. This project also resulted in the generation of two Master's theses and one Ph.D. thesis.

2010 Environment and Natural Resources Trust Fund (ENRTF) Work Program Final Report

Date of Report: August 22, 2013
Final Report
Date of Work Program Approval: June 9, 2010
Project Completion Date: June 30, 2013

I. PROJECT TITLE: Ecological Impacts of Effluent in Surface Waters and Fish

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Location: Minneapolis, Minnesota 55455 or St. Cloud, Minnesota 56301; Additional work (sampling) will take place in Mankato, Minnesota and Brewster, Minnesota. See attached map.

Total ENRTF Project Budget:	**ENRTF Appropriation $**	**340,000**
	Minus Amount Spent: $	**340,000**
	Equal Balance: $	**0**

Legal Citation: M.L. 2010, Chp. 362, Sec. 2, Subd. 5c

Appropriation Language:
$340,000 is from the trust fund to the Board of Regents of the University of Minnesota in cooperation with St. Cloud State University to determine the chemical and biological fate of phytoestrogens in surface waters and the impacts on fish. This appropriation is available until June 30, 2013, by which time the project must be completed and final products delivered.

II. FINAL PROJECT SUMMARY AND RESULTS:

Phytoestrogens are plant-based compounds that mimic estrogen and can interfere with normal biological development. Research shows that phytoestrogens are discharged into surface water from wastewater treatment plants and certain industries. The biological effects of these compounds have not been well studied, although it is known that they can feminize male fish. Almost nothing is known about their environmental fate. When these compounds enter rivers and streams, it is likely that they will be

degraded and therefore may have a lessened impact on biota, but this needs to be confirmed

In this project, the persistence of two common phytoestrogens (genistein and daidzein) was studied. Fathead minnow exposure experiments at realistic environmental concentrations were also performed. Experiments demonstrated that genistein and daidzein reacted with sunlight. These two compounds also biodegraded rapidly in natural water samples; the rate of degradation depended on phytoestrogen concentration, water/incubation temperature, and the source of the water. Sorption experiments showed that phytoestrogens sorb to sediment, but this is not likely to be an important loss mechanism. Adult fathead minnow exposure experiments showed that only subtle effects on anatomy, physiology and behavior of fathead minnows occurred as a result of exposure to phytoestrogens singly or in mixtures. The one exception to this was the fact that adult fathead minnows produced significantly more eggs when exposed to daidzein. Larval minnow exposures showed that exposure to genistein, formononetin (another common phytoestrogen), and a mixture of phytoestrogens had a negative impact on larval survival. Adult and larval exposures to microbiologically degraded phytoestrogens showed negative impacts on adult egg production. This research indicates that genistein, daidzein, and formononetin are unlikely to cause widespread ecological harm themselves in the absence of other stressors; nevertheless, caution should be exercised with respect to high concentration effluents due to the potentially anti-estrogenic effects of phytoestrogen degradates.

III. PROGRESS SUMMARY AS OF JANUARY, 2013:

Photolysis experiments with genistein and daidzein are complete and this work has been published. Biodegradation experiments are also complete and showed that genistein degradation was rapid, variable (based on initial concentration, temperature, and source water), and followed zero-order kinetics at high initial concentrations (100 µg/L) and first-order kinetics at lower initial concentrations (500 ng/L). Half-lives varied from about 18.5 to 53 hours. Although degradation was relatively rapid, phytoestrogens are likely to be more persistent in cold weather months from discharges to lower-flow streams, such as Okabena Creek. Laboratory-scale reactor experiments showed that although genistein degradation occurred under nitrifying conditions (low carbon, long residence time, high ammonia concentration), nitrifiers were not responsible for the transformation. This points to genistein degraders as multiple substrate utilizers that may be slow growers. Grab samples collected in the Minnesota River up- and downstream of the Mankato WWTP November 2011, May 2012, and June 2012 for showed that any phytoestrogens present in the effluent were fully diluted by the river upon discharge. Like the Minnesota River samples, samples collected up- and downstream of the Brewster WWTP in June of 2012 showed that the effluent did not impact Okabena Creek water quality with respect to phytoestrogens; no phytoestrogen signature from the Brewster WWTP discharge was discernable. Sorption isotherms for genistein, daidzein, and formononetin to sediment collected from the Mississippi River were determined, as were isotherms for the sorption of genistein and biochanin A to

kaolinite and Na- and Ca-montmorillonite at a range of pH values. Preliminary modeling to understand some of the mechanisms of sorption was also performed. Adult fathead minnow exposure experiments suggested only subtle effects (on anatomy, physiology and behavior of mature fathead minnows) as a result of exposure to phytoestrogens singly or in mixtures. Larval exposures, however, showed that genistein, daidzein, a mixture of phytoestrogens, sediment-sorbed genistein, and sediment-sorbed formononetin all had negative impacts on larval survival, although no impacts on larval predator avoidance were observed.

Amendment Request (01/20/2012):
The addendum is to formally request a re-budgeting of funds for this project.

As part of the project, an accelerated solvent extraction system is used to extract phytoestrogens from sediment for modeling and sorption studies. A valve in the system has broken and needs to be replaced. While the part is not under warranty, we have negotiated a reduced cost for the repair, because this is something that should not have failed so soon. The total cost of the repair has been quoted at $2,300. Based on usage to date, 70% of the repair ($1610) will be charged to this project (the remaining funds for the repair will be obtained from a requested re-budget of Prof. William Arnold's LCCMR project, M.L. 2010 5f - Evaluation of Dioxins in Minnesota Lakes, which co-purchased and is co-using the instrument). We would like to rebudget $2,000 from the "**Personnel**" category to the "**Equipment/Tools/Supplies**" category where "and instrument maintenance and repair costs" has been added. The additional funds being re-budgeted are insurance against any future repairs.

In addition, personnel requirements for Result 2 (Determine the impact of the phytoestrogens on fathead minnows) are less than originally anticipated because undergraduate students have been able to complete more of the work that was proposed. The supply costs for determining phytoestrogen concentrations in rivers downstream of the wastewater treatment plants are higher than originally anticipated because of the low concentrations of these compounds as a result of dilution from the high water levels this past spring and summer. Because of this a rebudget of $15,000 is requested from the "**Personnel**" category to the "**Equipment/Tools/Supplies**" category.

The movement of money between categories will not affect project objectives or timelines.

Amendment Approved: 01/24/2012

Amendment Request (07/31/2012):
The addendum is to formally request a re-budgeting of funds for this project.

Result 2 is primarily being conducted at St. Cloud State University under the direction of Heiko Schoenfuss (co-investigator). A graduate student hired through the University of Minnesota has been working on Result 2, but an additional student with specific training

in histological methods was required to complete this research. Therefore, we would like to rebudget $24,000 from the "**Personnel**" category (student support for Result 2) to the "**Subcontract**" category, where it will be used for the exact same purpose (student support for Result 2) with the only difference being that the student will attend St. Cloud State University rather than the University of Minnesota.

The movement of money between categories will not affect project objectives or timelines.

Amendment Approved: 08/2/2012

Amendment Request (04/3/2013):
The addendum is to formally request a re-budgeting of funds for this project.

The supply costs for determining phytoestrogen concentrations in rivers and performing experiments on the biological and chemical fate of phytoestrogens have been higher than originally anticipated because of the low concentrations of these compounds and the large number of experiments performed. This has resulted in additional analytical and material costs. Because of this a rebudget of $14,000 is requested from the "**Personnel**" category (Result 2) to the "**Equipment/Tools/Supplies**" category (Result 1).

The movement of money between categories will not affect project objectives or timelines.

Amendment Approved: 04/5/2013

IV. OUTLINE OF PROJECT RESULTS:

RESULT 1: *Determine the chemical and biological fate of phytoestrogens in surface waters*

Description:
We know that phytoestrogens are discharged to surface water from municipal wastewater treatment plants and from industrial facilities (including some dairies, meat processors, peanut processors, and soy processors). Nevertheless, no research has been conducted on their biological or chemical fate in the environment. It is likely that these compounds will adhere to particles in the receiving water and will undergo chemical and biological reactions. These processes will control the concentration of the phytoestrogens, and therefore, their ecological effect (see Result 2, below). Laboratory experiments will be performed with the two most-commonly observed phytoestrogens: genistein and daidzein. Single compounds and mixtures will be added to river water samples collected downstream of two soy-processing facilities in Minnesota (in Mankato and Brewster). The biological transformation of the phytoestrogens will be measured with time using liquid chromatography/mass spectrometry under different conditions

(phytoestrogen concentration, biomass levels, oxygen levels). The estrogenicity of any byproducts formed will be determined as well, using a yeast estrogen screen assay. Experiments to determine photolysis (sunlight-driven reactions) rates of genistein and daidzein will also be conducted in river and pure water in both artificial and natural light. The effect of naturally-occurring ions and organic matter on photolysis rates will be investigated. Again, the estrogenicity of the byproducts will be determined. Finally, the water-solid partitioning coefficients will be determined for both compounds.
After quantifying the appropriate rate constants, verification of the importance of these processes in the field is required. We will determine the concentration of genistein and daidzein at the point of discharge and in the rivers/streams downgradient of the two soy-processing facilities. A model for the concentration of phytoestrogens as a function of distance will be built, based on our experimental results, and compared to the concentrations measured in the field.

Summary Budget Information for Result 1:	**ENRTF Budget:**	**$**	**220,000**
	Amount Spent:	**$**	**220,000**
	Balance:	**$**	**0**

Deliverable/Outcome	**Completion Date**	**Budget**
1. Determine the biological transformation kinetics for genistein and daidzein	6/30/12	62,000
2. Determine kinetics of photolysis for genistein and daidzein	6/30/12	56,000
3. Determine estrogenicity of transformation products of genistein and daidzein	6/30/12	21,000
4. Measure the effluent concentrations and downgradient concentrations of genistein and daidzein in the field	9/30/12	40,000
5. Build and verify a model to determine the importance of various natural processes on phytoestrogen fate in the environment	4/30/13	41,000

Result Completion Date: April 30, 2013

Result Status as of January 2011:
The UV-visible absorbance spectra of genistein and daidzein have been measured and pK_a values have been determined. Genistein and daidzein have been found to undergo direct photolysis, and the quantum yields of direct photolysis for genistein and daidzein have been determined using natural sunlight and chemical actinometry (see Table 1 below). The direct photolysis of daidzein is unaffected by calcium and magnesium; the effect of these cations on genistein photolysis is currently being investigated.

Table 1. Quantum Yields of Direct Photolysis of Genistein and Daidzein

Genistein	Φ	Daidzein	Φ
pH 5	0.000003	pH 5	0.00043

pH 8.5	0.000038	pH 8.7	0.001
pH 11	0.000044	pH 12	0.00019
pH 12	0.00114		

Indirect photolysis for both compounds is currently being investigated as well. The rate constant for the reaction between daidzein and hydroxyl radical has been determined to be $1.01*10^{10}$; genistein has not yet been investigated.

Several biodegradation experiments have been performed with surface water that is unimpacted by soy-processing facilities. The biodegradation rates of genistein and daidzein are extremely variable in surface water, with 90% of the parent compound degrading in approximately 2-7 days. The rate of degradation appears to depend on several factors including water temperature, biomass concentration, water turbidity, and phytoestrogen concentration. Additional experiments are currently being performed to better-understand the influence of various environmental parameters. Experiments using surface water from soy processing-impacted areas at Brewster and Mankato, MN are planned for this summer.

Result Status as of July 2011:

The reaction rates between genistein and daidzein and reactive oxygen species (ROS) have been determined (see below).

Rate constants for reactions between genistein and daidzein and ROS

	k_{OH} (M^{-1} s^{-1})	K_{1O2} (M^{-1} s^{-1})
Genistein	8.73×10^{9}	3.57×10^{7}
Daidzein	~~1.01×10^{10}~~ 6.9×10^{9}	1.84×10^{7}

Sampling downgradient of the Mankato wastewater treatment plant (WWTP) was postponed until late June because of high water. On June 20th the sampling trip was made and samples were taken to perform biodegradation and sediment sorption experiments in the laboratory at the University of Minnesota. Samples were also taken to monitor the attenuation of phytoestrogens downstream from their input at the WWTP effluent discharge.

Experiments designed to understand the sorption of genistein and daidzein to solid matrices have also begun using both natural sediments (collected from the Minnesota River upstream of the Mankato WWTP) and model solids (*e.g.,* kaolinite). Preliminary data suggests that genistein and daidzein do not sorb appreciably to kaolinite. Additional experiments will explore the effect of kaolinite concentration on sorption. Preliminary experiments performed with the river sediment show that sorption increases as pH decreases.

Biodegradation experiments were performed with the Mankato surface water. In order to begin to study those environmental parameters that impact degradation, experiments were performed with varying initial genistein concentrations (150 ng/L, 1 μg/L and 100

μg/L). Dissolved oxygen and pH were also monitored. Degradation of genistein was rapid and zero order over this range of concentrations, with genistein dropping below the detection limit (1.5 ng/L) within 70 hours.

Twenty-five grab samples were collected from the Mankato WWTP effluent and at select locations downstream of the wastewater outfall. These samples were collected to determine the effects of dilution/degradation on the concentration of various phytoestrogen species. A method is currently being refined on the LC-MS/MS to analyze for genistein, diadzein, zearalenone, formenonetin, coumestrol, and biochanin A at the Masonic Cancer Center.

Result Status as of January 2012:

Grab samples collected during the June 20th, 2011 sampling trip were analyzed for six phytoestrogens: zearlenone, daidzein, coumestrol, formenonetin, genistein and biochanin A. Samples collected upstream of the Mankato WWTP yielded the following phytoestrogen concentrations: zearlenone (1.1 ng/L), daidzein (1.8-1.5 ng/L), formenonetin (2.4-1.9 ng/L), genistein (1.8-1.6 ng/L) and biochanin A (1.9-1.6 ng/L). Analysis of the downgradient samples showed no impact from the Mankato WWTP on phytoestrogen concentrations in the Minnesota River (essentially the same concentrations of phytoestrogens were detected upstream and downstream of the wastewater treatment plant). It is likely that this is a result of the Minnesota River being near flood stage (22 ft) during sampling and the subsequent dilution of the wastewater effluent that occurred. The high water level also made collecting a direct WWTP effluent sample impossible (the outlet was under the water level) and therefore the degree to which the effluent was diluted by the river could not be determined. The closest sample was collected within 10 ft of the effluent pipe and the furthest downstream sample collected was approximately 0.6mi downstream of the WWTP.

A second sampling trip was conducted in November 2011. Thirteen grab samples were collected from the Minnesota River at Mankato (1 upstream, 1 effluent, and 11 downstream samples) and 10 samples were collected up- and downstream of the Brewster WWTP. These samples have been prepared for analysis by LC-MS/MS, which will take place in March at the Masonic Cancer Center at the University of Minnesota. Water was also collected from both locations to conduct biodegradation experiments in the laboratory.

Batch genistein biodegradation experiments were conducted using water collected from the Minnesota River at Mankato (collected in November 2011). Genistein was added to the water at concentrations of 100 μg/L and 150 ng/L and degradation was monitored over time. Degradation of genistein when added at 100 μg/L was zero-order, with reaction rate coefficients of 0.0644-0.0183 μg/L-hr. Analysis of the genistein degradation at 150 ng/L study is on-going. Biodegradation experiments will be conducted at 10°C using Minnesota River water to determine the effect of temperature on biodegradation rates. Experiments will be conducted with the water collected from Brewster following an identical protocol.

The photolysis experiments have been completed and this work has been submitted to *Environmental Science & Technology* for publication. No effect of calcium or magnesium ions on genistein photolysis was seen. It was shown that reaction with triplet-state natural organic matter is the major photolytic reaction pathway for genistein and reaction via direct photolysis and with singlet oxygen are the major photolytic reaction pathways for daidzein. Additionally, the second-order rate constant for the reaction of daidzein with hydroxyl radical was determined to be 6.9×10^9 $M^{-1}s^{-1}$ (note, this value was incorrectly reported in the July 2011 report, now corrected with a mark-out). Under certain conditions where hydroxyl radical is present in high concentrations, reaction with hydroxyl radical could be important for both compounds. Subject only to direct and indirect photolysis, genistein is expected to have a half-life on the order of days to weeks, while daidzein is expected to have a half-life on the order of hours to days.

Sorption experiments are ongoing. It was shown that both compounds do sorb to kaolinite, but the amount sorbed varies with the initial concentration. It was also shown that pH affects the amount sorbed over a range of initial concentrations. Cation bridging and electrostatic interactions are expected to be important mechanisms of sorption to clays, and experiments are being conducted to assess their importance for sorption to kaolinite and montmorillonite. Sorption experiments with iron oxides are also planned.

Result Status as of July 2012

Grab samples collected upstream and downstream of the Mankato and Brewster WWTPs during the November 2011 sampling trip were analyzed. Samples collected upstream of the Mankato WWTP yielded the following phytoestrogen concentrations: zearlenone (1.1-1.2 ng/L), daidzein (1.8-1.5 ng/L), formenonetin (2.4-1.9 ng/L), genistein (2.6-1.7 ng/L) and biochanin A (2.3-1.7 ng/L). Analysis of the downgradient samples showed no impact from the Mankato WWTP on phytoestrogen concentrations in the Minnesota River (essentially the same concentrations of phytoestrogens were detected upstream and downstream of the wastewater treatment plant). Again, although not at flood stage, it is likely that the effluent is sufficiently diluted by the Minnesota River such that no impact of the wastewater effluent on the river is seen. Interestingly, these values are very similar to that seen in June 2011, suggesting that the background phytoestrogen concentrations in the Minnesota River at Mankato are fairly stable. Unfortunately, during processing, the Brewster samples were lost and could not be analyzed. Samples were also taken June 6th, 2012 (13 at Mankato and 12 at Brewster) and have been processed for analysis. These samples will be analyzed on the LC-MS/MS at the Masonic Cancer Center (University of Minnesota) in August (2012).

Experiments to determine the sorption isotherms and edges for genistein and daidzein to kaolinite, montmorillonite, and goethite, three common soil minerals, have been performed. The isotherms allowed the calculation of the distribution coefficient, K_D, for each combination, which are shown below.

K_D values (L/kg, with 95% confidence intervals) for phytoestrogens on soil minerals

	Ca-montmorillonite (pH 8.5)	Na-montmorillonite (pH 8.5)	Kaolinite (pH 8.5)	Goethite (pH 7.1)
Genistein	--	130 ± 220	64 ± 15	253 ± 60
Daidzein	40 ± 16	--	44 ± 31	37 ± 8

The edge experiments, in which the concentration is held constant and the pH is varied, showed that both genistein and daidzein show a sorption maximum on goethite near pH 7, with slightly lower sorption at lower pH values and steadily decreasing sorption as pH increases. With kaolinite, daidzein shows minimal sorption above pH 7 (K_D ~ 30 L/kg), with increasing sorption with decreasing pH, up to K_D ~ 70 L/kg at pH 3.5. Genistein does not sorb above pH 10, but sorption increases with decreasing pH.
Daidzein displays weakly pH-dependent sorption on montmorillonite, but at all pH values tested, higher concentrations of $CaCl_2$ increased the sorption. Increased concentration of NaCl had a weaker effect than $CaCl_2$. With Na-montmorillonite, genistein showed increasing sorption with increasing pH, while Ca-montmorillonite showed a weak pH effect, but a marked increase in sorption with the highest concentration of $CaCl_2$ (0.5 M) compared to lower concentrations (0.1 M and 0.01 M). Interpretation of the sorption edges, including modeling, are in progress and sorption experiments using organic matter-amended soil minerals as well as natural sediments are planned.

Experiments determining the biological degradation rates of genistein were conducted using water collected from the Minnesota River at Mankato, and the Okabena Creek at Brewster. Variation in the degradation rates of genistein based on concentration, temperature, source water, and season were determined. These rates are summarized below.

Degradation rate coefficients for genistein degradation when fed genistein at an initial concentration of 500 ng/L

				Zero Order Kinetics			First Order Kinetics		
Source[1]	**Water Collection Date**[2]	**Reactor #**	**Temp [°C]**[3]	**K [µg/L-hr]**	**R^2**	**Average ± 95% CI [µg/L-hr]**	**K [1/hr]**	**R^2**	**Average ± 95% CI [1/hr]**
MN River, Mankato, MN	6/6/12	1	20	7.60 E-03	0.827	$7.00 \times 10^{-3} \pm 2.42 \times 10^{-3}$	-2.98E-02	0.998	$3.39 \times 10^{-2} \pm 1.76 \times 10^{-3}$
		2		8.20 E-03	0.798		-3.60E-02	0.992	
		3		5.19 E-03	0.707		-3.76E-02	0.997	
MN River, Mankato, MN	5/14/12	1	20	3.48 E-03	0.944	$4.88 \times 10^{-3} \pm 5.64 \times 10^{-4}$	-1.83E-02	0.976	$2.24 \times 10^{-2} \pm 1.28 \times 10^{-3}$
		2		5.72×10^{-3}	0.951		-2.52E-02	0.995	
		3		5.43×10^{-3}	0.959		-2.38E-02	0.987	
MN River, Mankato, MN	6/6/12	1	10	1.86 E-03	0.786	$2.07 \times 10^{-3} \pm 6.25 \times 10^{-4}$	-2.03E-02	0.98	$1.76 \times 10^{-2} \pm 1.71 \times 10^{-3}$
		2		2.33 E-03	0.901		-1.93E-02	0.991	
		3		2.01 E-03	0.955		-1.30E-02	0.994	
Okabena Creek, Brewster, MN	6/6/12	1	20	7.17 E-03	0.929	$7.96 \times 10^{-3} \pm 1.79 \times 10^{-3}$	-1.62E-02	0.81	$2.11 \times 10^{-2} \pm 6.99 \times 10^{-3}$
		2		9.93 E-03	0.877		-2.72E-02	0.818	
		3		6.77 E-03	0.89		-2.00E-02	0.771	

[1] Source refers to either the Minnesota River at Mankato, MN or Brewster, MN where bulk water was collected and subsequently used to run batch reactors
[2] Water collection date is the date of bulk water collection
[3] Reactors were either run at 10 or 20 °C, which is indicated by the temperature column

Degradation rate coefficients for genistein degradation when fed genistein at an initial concentration of 100 µg/L

				Zero Order Kinetics			First Order Kinetics		
Source	**Water Collection Date**	**Reactor #**	**Temp [C]**	**K [µg/L-hr]**	**R^2**	**Average ± 95% CI [µg/L-hr]**	**K [1/hr]**	**R^2**	**Average ± 95% CI [1/hr]**
MN River, Mankato, MN	5/14/2012	M1	20	6.83	0.971	$6.08 \pm 7.68 \times 10^{-1}$	-2.78E-01	0.839	$2.31 \times 10^{-1} \pm 7.48 \times 10^{-2}$
		M2		5.59	0.98		-1.72E-01	0.879	
		M3		5.81	0.977		-2.43E-01	0.868	
MN River, Mankato, MN	11/8/2011	M1	20	2.32	0.964	2.86 ± 1.12	-4.99E-02	0.837	$9.37 \times 10^{-2} \pm 2.34 \times 10^{-1}$
		M2		4.21	0.935		-9.49E-02	0.798	
		M3		2.04	0.971		-5.46E-02	0.767	
Okabena Creek, Brewster, MN	11/8/2011	B1	20	2.29	0.635	2.92 ± 2.07	-2.19E-02	0.599	$1.29 \times 10^{-1} \pm 1.83 \times 10^{-1}$
		B2		3.20	0.918		-1.63E-01	0.624	
		B3		3.29	0.908		-1.75E-01	0.604	

Temperature, source water and season affected the degradation rate by as much as 100%, whereas initial genistein concentration affected rates less dramatically. High concentration experiments (100 µg/L) produced a variable lag period followed by rapid, near zero-order degradation. Half-lives varied from about 30 to 51 hours. Low concentration experiments (0.5 µg/L, or 500 ng/L) were best modeled using first order kinetics; half-lives in these experiments ranged from 18.5 to 53 hours. Overall, genistein appears to be readily degradable with highly variable rates. Our data also suggests that phytoestrogens are likely to be more persistent in cold weather months from discharges to lower-flow streams, such as Okabena Creek.

The following manuscript has been published:

Kelly MM, Arnold WA. 2012. Direct and Indirect Photolysis of the Phytoestrogens Genistein and Daidzein. *Environmental Science and Technology*, 46(10):5396-5403.

Result Status as of January 2013

All grab samples collected upstream and downstream of the Mankato (June 2011, November 2011, and May 2012) and Brewster WWTPs (June 2012) have been analyzed. Even downstream of these suspected phytoestrogen sources, the phytoestrogens monitored in this study were only detected in the low nanogram per liter range. In Minnesota River water sampled on 6/20/11, all species, with the exception of coumestrol, were present; in the Minnesota River samples taken on 11/8/11, only one sample, the WWTP effluent from the city of Mankato, contained phytoestrogens (daidzein and formononetin). Samples from Okabena Creek and the Brewster WWTP effluent showed a similar pattern, with only genistein and daidzein detected in the low nanogram per liter range. These results again indicate that the ability to biodegrade genistein (and other phytoestrogens) is likely to be ubiquitous in the environment. This is supported by the lack of high genistein concentrations (and phytoestrogens in general) in samples downstream of probable sources.

Degradation rate coefficients for genistein under a variety of conditions are given in the July 2012 result status section. Confidence intervals were recalculated using an alternative (yet equally accepted) technique; a number of other errors were found and corrected. These are all shown as corrected with a mark-out. As mentioned above, genistein was found to degrade both readily and variably in surface water samples, with differences in degradation rates based on source water, month of sample collection, and incubation temperature.

Laboratory-scale reactor studies with a highly enriched culture were performed to determine which organism(s) were responsible for genistein degradation. Results showed that although the culture was developed under nitrifying conditions in the absence or genistein, the organisms present were able to readily degrade genistein. This supports the notion that genistein degraders could be multiple substrate utilizers. In addition, the addition of an inhibitor of nitrification, allylthiourea, halted ammonia and nitrite oxidation in the reactor, but did not stop or slow genistein degradation. This demonstrates that nitrifiers are not likely to be responsible for genistein degradation.

Therefore, the higher genistein removal rates observed by others in WWTPs that employ tertiary treatment may not be a direct result of nitrifying organisms, but rather to other microorganisms enriched under similar conditions.

The biodegradation results are currently being prepared for publication.

Experiments to determine the sorption isotherms for genistein, daidzein, and formononetin to sediment collected from the Mississippi River were performed, using well water from the SCSU lab. Formononetin had a K_D of 157, genistein had a K_D of 58, and daidzein had a K_D of 12. Isotherms for the sorption of genistein to Ca-montmorillonite were also completed at pH 6, 8.5, and 11. The K_D values were 72, 61, and 128, respectively. Isotherms for the sorption of genistein to Na-montmorillonite were completed at pH 6 and 11. The K_D values were 100 and 50, respectively.
Isotherms for the sorption of biochanin A to kaolinite, Na- and Ca-montmorillonite were also completed, showing K_D values of 1200, 82 and 1310, respectively.
Preliminary modeling efforts of the previously described sorption edges suggest that one important factor in determining the extent of sorption of genistein or daidzein to goethite is the prevalence of protonated surface sites. With respect to kaolinite sorption, although kaolinite is thought to have variable surface charge than goethite, the more important factor for sorption to kaolinite seemed to be the fraction of genistein or daidzein in the fully protonated form.

Final Report Summary:
Photolysis: UV-visible spectra for genistein and daidzein at varying pH values were taken, the pK_a values for both compounds were measured, and UV-visible spectra for each protonation state were determined. The loss of both compounds in deionized water was observed upon exposure to natural sunlight, and the quantum yields were determined for each protonation state. In Mississippi River water, direct photolysis does not account for all of the loss of genistein and daidzein. The mechanism of indirect photolysis was probed, and results suggest that daidzein is transformed mainly via direct photolysis and singlet oxygenation, while genistein is transformed mainly via reaction with triplet-state natural organic matter. These results have been incorporated into one manuscript that has been published in *Environmental Science and Technology* (included with the final report documents).

Sorption: K_d values (sorption coefficients) were obtained by sorption isotherms for genistein, daidzein, formononetin, and biochanin A on several clays and iron minerals (goethite, kaolinite, Ca- and Na-montmorillonite) and sediment collected from the Mississippi River. Biochanin A showed the highest K_d values for sorption to kaolinite and Ca-montmorillonite. Genistein exhibited the next highest K_d values for sorption to goethite at low pH values where it is mostly protonated and the goethite has protonated surface sites. Daidzein did not sorb strongly to any of the tested sorbents. When montmorillonite was the sorbent, genistein showed no obvious relationship between K_d and ion composition. For biochanin A and daidzein, however, sorption to Ca-montmorillonite was stronger than to Na-montmorillonite. Genistein and daidzein both clearly displayed pH-dependent sorption to goethite and all of the tested phytoestrogens

exhibited pH-dependent sorption to kaolinite. With respect to Mississippi River sediment, the K_d values were converted to log K_{oc} values (the sorption to organic carbon), yielding 3.88 for genistein, 3.51 for daidzein, and 4.26 for formononetin. These can therefore be considered to be moderately sorptive compounds. Compared to traditional persistent organic pollutants, isoflavones do not sorb especially strongly to clay minerals, iron oxides, or natural sediments. Nevertheless, if high concentrations or frequent discharges of isoflavones are released to natural waters, sorption to settling particles will play a role in the attenuation of these compounds. The role of sorption in the total attenuation of phytoestrogens will depend on sediment composition, as well as non-sorptive factors such as time of day and dissolved organic matter composition, which influence removal by photolysis and biological activity.

Using this data, the sorbed fraction of genistein and daidzein in a hypothetical lake were calculated. In a lake at pH 8.5 and 0.01 M NaCl with 5 mg/L of suspended sediment, composed of 50% quartz, 10% goethite, 10% kaolinite, 10% Na-montmorillonite, and 20% organic matter, 0.77% of the genistein and 0.32% of the daidzein would sorb to particles. Under the same conditions, but with 25 mg/L suspended sediment, 3.8% of the genistein and 1.6% of the daidzein introduced to the lake would sorb to particles. Assuming a typical settling velocity for suspended particles, 1.9% of the genistein and 0.8% of the daidzein would settle with particles every day. Therefore, sorption to particles may be important for aquatic systems receiving inputs of isoflavones in which the water moves very slowly. These results have been incorporated into one manuscript that will be submitted shortly (August/September 2013) for publication in the peer-reviewed literature.

Biodegradation: The persistence of genistein and daidzein in natural aquatic systems was assessed in riverine samples. Initial concentration, temperature, sample location, and time of sample collection were varied. Genistein and daidzein were found to be readily biodegradable at all tested concentrations (0.5 to 100 µg/L), at both 10 and 20°C, in samples collected in different seasons, and in samples from three different rivers. Genistein degradation appeared to be performed by heterotrophic bacteria capable of scavenging a range of low-concentration carbonaceous compounds for survival. These results suggest that there is minimal risk of the presence of high phytoestrogen concentrations in receiving waters if at least some wastewater treatment is provided at point sources. Nevertheless, caution and more research should be focused on phytoestrogen persistence at low temperatures, during which degradation rates drop and these compounds could build-up in the water column or in sediment and impact aquatic wildlife as a result. These results have been incorporated into one manuscript that has been submitted for publication in the peer-reviewed literature (included with the final report documents).

Modeling and overall assessment: With an understanding of the chemical properties and biodegradability of genistein and daidzein developed, it was possible to predict the environmental fate of these two compounds under specific environmental conditions. To do so, a box model representing a lake and a multi-box model representing a stream or river were developed (inputs given below in the table).

Table 1. Parameters used as inputs to the box model of a lake

	Summer, GEN	Winter, GEN	Summer, DDZ	Winter, DDZ
Volume (m^3)	1.50×10^8	1.50×10^8	1.50×10^8	1.50×10^8
Concentration of phytoestrogens leaving the lake (ng/L)	1.50×10^6	1.50×10^6	9.8×10^5	9.8×10^5
Flow of water entering and leaving the lake (m^3/day)	3.40×10^5	3.40×10^5	3.40×10^5	3.40×10^5
Fraction of effluent entering the lake	0.1	0.1	0.1	0.1

The models shown describe the concentration of genistein or daidzein in the lake in a scenario where a treatment plant discharging to the lake begins accepting waste from a soy-processing facility, resulting in an effluent concentration of 150,000 ng/L genistein and 98,000 ng/L daidzein at a flow rate of 3.40×10^4 m^3/day. The steady-state concentration reached for each scenario is given in the table below.

Table 2. Steady-state concentrations for genistein and daidzein in summer and winter in a model Lake

	Summer, GEN	Winter, GEN	Summer, DDZ	Winter, DDZ
No removal	1.50×10^4	1.50×10^4	9.80×10^3	9.80×10^3
Photolysis only	5.77×10^3	6.37×10^3	2.00×10^2	4.13×10^2
Biodegradation only	0	0	0	0
Sedimentation only	1.50×10^4	1.50×10^4	9.78×10^2	9.78×10^2
All processes	0	0	0	0

To evaluate the persistence of genistein and daidzein in streams, a multi-box model was constructed. Two streams were imagined, one (“Minnesota River”) with a higher flowrate (20,000 m^3/hr) and lower fraction of effluent entering the stream (0.05), and one (“Zumbro River”) with a lower flowrate (500 m^3/hr) and a higher fraction of effluent entering the stream (0.4). The volume of each box was set to assume a cross sectional area of 10 m^2, and the length of the box was set to 10 m. It was assumed that each box came to equilibrium within its residence time, Q/V. When this model was evaluated, it was seen that biodegradation alone controlled genistein and daidzein persistence and did not allow either compound to accumulate. Direct photolysis of daidzein appears to speed the rate of loss very slightly.

From these models we see quite clearly that where biodegradation of concentrated effluents is occurring, genistein and daidzein will not accumulate. Even at sites where biodegradation is very slow, direct and indirect photolysis efficiently degrade genistein and daidzein, particularly in the summer months, and could hold the concentrations in a lake below the biological threshold of 1000 ng/L. Nevertheless, because the biodegradation products of genistein and daidzein have been shown to possess androgenic or anti-estrogenic activity (see Result 2 below), aquatic organisms could still be impacted. To verify these modeling results, samples were taken downstream from two wastewater discharges; very low phytoestrogen concentrations were observed (low ng/L levels). The field sampling results have been incorporated into a manuscript that has been submitted for publication in the peer-reviewed literature (included with the final report documents).

RESULT 2: ***Determine the impact of the phytoestrogens on fathead minnows***

Description:
The fathead minnow will be used as the biological model for this research, as this organism is used as a screening organism for EDCs by the US EPA and it is an important component of the Minnesota aquatic food chain. While two previous studies have indicated behavioral and physical changes in phytoestrogen-exposed fish, these studies did not use realistic compound concentrations, mixtures, or fish native to Minnesota. We propose to assess the effects of genistein and daidzein (singularly and in mixtures) over a range of environmentally-relevant concentrations in controlled laboratory experiments. Three life stages (embryo, larva, adult) of the fathead minnow will be investigated to assess developmental, behavioral, and physical changes (including feminization) in the fish. For each life stage, fish will be exposed to three concentrations of each compound (spanning observed environmental concentrations) and to three mixtures of the two compounds. Following exposure, embryos and larvae will be assessed in their ability to perform innate predator avoidance behaviors. Adult fathead minnows (males and females) will be assessed for changes in their reproductive behavior. Fish will also be analyzed for vitellogenin concentrations (a precursor protein involved in egg production and a sign of feminization of male fish) and their livers and reproductive organs will be evaluated for changes. Finally, we will also perform in-stream experiments downstream of the discharge of the two soy-processing facilities to verify the results of laboratory experiments.

Summary Budget Information for Result 2:	**ENRTF Budget:**	**$**	**120,000**
	Amount Spent:	**$**	**120,000**
	Balance:	**$**	**0**

Deliverable/Outcome	Completion Date	Budget
1. Determine the effects of phytoestrogens on embryonic and larval fathead minnow behavior	4/30/11	49,000

2. Determine the effects of phytoestrogens on the reproductive behavior of mature fathead minnows	4/30/12	42,500
3. Determine the effects of phytoestrogens on the physiology of mature fathead minnows (feminization, liver, and reproductive organ changes)	4/30/13	28,500

Result Completion Date: April 30, 2013

Result Status as of January 2011:

To determine the biological effects range of three common phytoestrogens, we exposed groups of 20 males and 20 females each to one of three phytoestrogens (Daidzein; Genistein, Formononetin) at 1,000 ng/L or mixtures of the three compounds at a combined concentration of 1,000 ng/L or 3,000 ng/L for 21 days. An ethanol carrier control and a 50 ng/L 17ß-estradiol positive control were added for quality control purposes. Immediately following exposure, 10 males/10 females from each treatment were sacrificed for analysis of secondary sex characteristics, relative size of gonads and livers, plasma vitellogenin concentrations, and histopathology. The remaining ten males/females per treatment were assessed in an aggression behavioral assay and then also sacrificed and assessed as described above.

We have completed the exposure experiment, and have completed histological and vitellogenin analysis and are currently conducting statistical analysis of the collected data sets. Water samples have been forwarded to the Novak laboratory for analysis.

Result Status as of July 2011:

We have completed the analysis of the first complete exposure experiment of fathead minnows in a flow-through exposure system to a suite of phytoestrogens and their mixtures. All biological data have been gathered (including vitellogenin analysis and histopathology) and statistically analyzed. The results indicate a weak, but statistically significant induction of plasma vitellogenin in male fish exposed to daidzein at 1 μg/L and the mixture of all three phytoestrogens (daidzein, genistein, and formononetin) at 1 μg/L each (Figure 1).

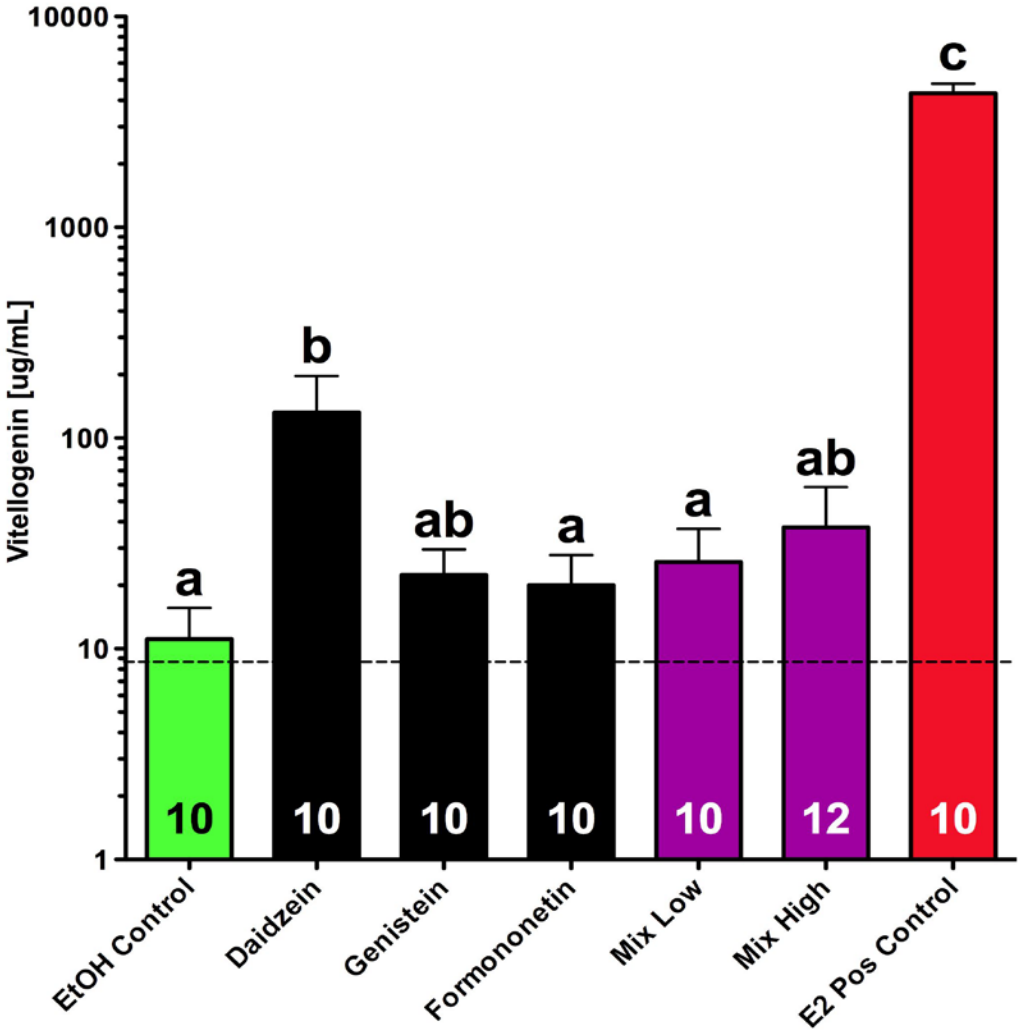


Figure. Male plasma vitellogenin concentrations (ug/mL) after 21-day flow-through exposure to phytoestrogens individually and in mixture.

Statistical analysis based on log transformed vtg data. ANOVA with Tukey's post test ($p<0.0001$). Letters indicate significant differences between treatments at $p<0.05$; numbers in each column indicate sample size. Dotted line indicates ELISA assay detection limit.

The results gathered from the first 21-day exposure study are being validated through a second experiment using additional reproductive endpoints. This exposure begun recently and will be completed by the end of August 2011.

Result Status as of January 2012:

We have completed two 21-day flow-through exposure experiments of male and female fathead minnows to three phytoestrogens at environmentally relevant concentrations (1,000 ng/L each of daidzein, genistein, formononetin). In each of these duplicate experiments we also included mixture treatments combining all three phytoestrogens at 330 ng/L each or 1,000 ng/L each. Finally, the duplicate experiments contained a

negative ethanol carrier control and a positive (50 ng/L) estradiol control. Each treatment consisted of two aquaria of 10 male fish each and two aquaria of 10 female fish each.

Immediately following exposure, we sacrificed 10 males and 10 females from each treatment for analysis of secondary sex characteristics, relative size of gonads and livers, plasma vitellogenin concentrations, and histopathology. The remaining ten (or less) fish per treatment were paired one male, one female and placed into the behavioral assay setup. These fish pairs (of the same previous exposure treatment) were then assessed in the aggression and reproduction assays for the following 14 days. We checked each reproductive pair daily for egg production with any deposited eggs being removed and counted to assess female fecundity. Egg viability was assessed three days after egg deposition by counting those eggs that contained eye-spots. All fertilized eggs were maintained until hatching to enumerate hatching success in each treatment.

All analysis of plasma samples for vitellogenin and liver and gonad samples for histopathology has been completed and results again indicate that feminization is occurring for phytoestrogen exposure. Data sets are currently been subjected to statistical analysis. In addition, we are developing an experimental design for a follow-up experiment to assess the effects of phytoestrogen metabolic breakdown products on mature male and female fathead minnows. We plan to conduct this experiment in spring 2012 with analysis completed by summer 2012.

Result Status as of July 2012

A third 21-day flow-through exposure experiment was conducted to compare the effects of phytoestrogens and their biological degradation products on fathead minnow anatomy, physiology, behavior and reproduction. We compared the same phytoestrogen mixture (1000 ng/L) used in the two previous flow-through exposure experiments with this same phytoestrogen mixture after biodegradation in a biologically active reactor (referred to as the “bioreactor”). The bioreactor (4 L) was modeled after a WWTP. Influent and effluent flows were 3 L/day. Media was made daily, to which phytoestrogens (500 ug/L each of formenonetin, genistein, and daidzein) were added. The bioreactor operated on a 14-day solids retention time. Aeration provided dissolved oxygen above 4 mg/L during the experiment. Samples were taken of the influent and effluent of the reactor and analyzed for pH, dissolved oxygen, ammonia, chemical oxygen demand, and suspended solids. Ten male and female baseline fish were dissected prior to exposure, with the equivalent number of males and females dissected after 21 days of exposure. Three treatments were compared: (1) the phytoestrogen mixture (positive control), (2) the bioreactor effluent containing degraded phytoestrogens, and (3) an ethanol control.

As in the two previous experiments, we sacrificed 10 males and 10 females from each treatment for analysis of secondary sex characteristics, relative size of gonads and livers, plasma vitellogenin concentrations, and histopathology immediately following

exposure. The remaining ten (or less) fish per treatment were paired one male, one female and placed into the behavioral assay setup. These fish pairs (of the same previous exposure treatment) were then assessed in the aggression and reproduction assays for the following 14 days. We checked each reproductive pair daily for egg production with any deposited eggs being removed and counted to assess female fecundity. Egg viability was assessed three days after egg deposition by counting those eggs that contained eye-spots. All fertilized eggs were maintained until hatching to enumerate hatching success in each treatment.

All analysis of plasma samples for vitellogenin and liver and gonad samples for histopathology has been completed and results are currently been subjected to statistical analysis.

Result Status as of January 2013

All adult replicate exposure experiments (see previous result status reporting) have been fully analyzed and suggest only subtle effects (on anatomy, physiology and behavior of mature fathead minnows) as a result of exposure to these compounds singly or in mixtures. Consequently we investigated the effects of these compounds on larval fathead minnows, a potentially more vulnerable life stage.

Three separate exposure experiments were conducted to compare larval fathead minnow escape performance, growth, and survival. The experiments were used to contrast un-degraded phytoestrogens (**compound exposure**), biodegraded phytoestrogens (presumably phytoestrogen degradation product exposure; **degraded exposure**), and phytoestrogens released from the sediment to which they were sorbed (**sediment exposure**). Larvae were exposed 21 days via 24-hour static renewal for the aqueous phytoestrogen exposures, and exchanged every 72 hours for the sediment experiment. Larval minnows (25) were randomly assigned to one of three exposure beakers providing 75 larvae per each treatment. Larval fish were fed 1 mL of live brine shrimp twice daily. Following exposure, a subset of 10 larvae from each beaker were assessed for predator avoidance performance following previously published protocols. Briefly, we transferred larvae individually to a testing arena overlaying a grid system and a vibrational stimulus. Using a high-speed camera and motioncamera software (Redlake), we recorded larval startle response. We measured body length (mm) latency (msec), velocity (BL/msec), and total escape response (BL/msec). At a later date, video files were analyzed using Image J. Remaining larvae were counted to use mortality as an endpoint. Temperature (ambient air and water), pH, and conductivity were measured daily and did not significantly differ between treatments in any experiment. Hach test strips were used to compare water chemistry every 4 days (total Cl, free Cl, total hardness, total alkalinity, and pH). No significant difference was found in any experiment.

Compound exposure. We compared six treatment groups: water blank, ethanol carrier, daidzein, formononetin, genistein, and a mixture (mixture high from earlier adult exposures). Survivors were enumerated after the escape performance assay. EtOH

survival was significantly higher than Genistein ($p<0.001$), Daidzein ($p<0.001$), and mixture exposure ($p=0.001$). No difference was found when comparing formononetin and EtOH survival ($p=0.12$).

Degraded exposure. We compared five treatment groups: degraded lake water control, degraded daidzein, degraded formononetin, degraded genistein, and degraded mixture (mixture high from earlier adult exposures). We found a significant difference in larval growth between daidzein and the mixture treatments ($p = 0.04$). Mean body length (mm) of fish exposed to the phytoestrogen mixture was lower than those exposed to daidzein. No other significance was detected.

Sediment exposure. We compared four treatment groups: sediment control, sediment daidzein, sediment formononetin, and sediment genistein. We did not see a significant difference on growth or escape performance. When comparing survival we found fish survival was higher for well water exposure relative to genistein ($p=0.002$) and formononetin ($p<0.001$). No significance was found for survival when comparing daidzein ($p=0.87$) to the well water control.

Final Report Summary:
All of the data was thoroughly analyzed in the context of controls and confirmational water chemistry data from the exposure experiments. The response of larval and sexually mature fathead minnows to environmentally relevant concentrations of three common phytoestrogens (genistein, daidzein, and formononetin) singly and in mixture was quantified. In addition, organismal responses in larval and sexually mature fathead minnows were quantified following exposure to microbiologically degraded phytoestrogens (genistein, daidzein, and formononetin). Larval survival was significantly reduced in genistein, formononetin and mixture treatments while adult male fish only exhibited subtle changes to their anatomy, physiology and behavior. Daidzein-exposed adult females produced significantly greater quantities of eggs. Products of the microbiological degradation of parent phytoestrogens did not have an effect on larval survival, growth, or predator avoidance. Female adult fathead minnows exposed to these degradation products produced significantly fewer eggs than those exposed to a control, but no other morphological, physiological, or behavioral changes were observed with male or female minnows. As phytoestrogens may bind to sediment, this route of larval exposure was also investigated, especially since larval fathead minnows remain in close contact with substrate for long periods of time. Larval fathead minnow exposures to sediments sorbed with genistein, daidzein, formononetin, and their mixtures through were conducted for 21-day exposure periods. After assessing larval survival growth and behavior in detail, no statistically significant decline compared to an unexposed control sediment was observed in any of the matrices. This research indicates that genistein, daidzein, and formononetin are unlikely to cause widespread ecological harm themselves in the absence of other stressors; nevertheless, caution should be exercised with respect to high concentration effluents due to the potentially anti-estrogenic effects of phytoestrogen degradates. These results have been incorporated into two manuscripts that have been submitted for publication in the peer-reviewed literature (included with the final report documents) and a third manuscript

currently under co-author review.

V. TOTAL ENRTF PROJECT BUDGET:

Personnel:	**$**	**187,000**
(William Arnold and Paige Novak, paid for 4% effort; 2 graduate students for approximately 1.75 years (averaging 29% effort over 3 years))		
Contracts:	**$**	**82,000**
(Some of the work will be conducted at St. Cloud State University (Result 2). The subcontract amount will include Co-PI salary (Heiko Schoenfuss, paid for 2.5% effort (less paid effort because of summer teaching commitments)), one undergraduate research assistant ($12,000 for 3 years), supplies for experiments (fish, etc., $36,000), and half of the funds required for travel to the site for sampling and in-stream experiments ($1,000)). Graduate research assistants hired and paid through UMN will work on both Result 1 and Result 2 and an additional graduate research assistant will be hired through St. Cloud State University to work on Result 2.)		
Equipment/Tools/Supplies:	**$**	**70,000**
(Laboratory supplies, analytical costs, and instrument maintenance and repair costs)		
Acquisition, including easements:	**$**	**0**
Travel:	**$**	**1,000**
(Travel to sites for sampling)		
Additional Budget Items:	**$**	**0**
TOTAL ENRTF PROJECT BUDGET:	**$**	**340,000**

Explanation of Capital Expenditures Greater Than $3,500: *None*

VI. PROJECT STRATEGY:

A. Project Partners: Dr. Paige Novak (University of MN), an expert in the occurrence of phytoestrogens and their biological transformation, will lead the project and coordinate the research. Dr. Heiko Schoenfuss (St. Cloud State University), an expert on the impact of endocrine disrupting compounds on aquatic biota, will direct the biological impact research. Dr. William Arnold (UMN), an expert in photolysis of endocrine disrupting compounds and building kinetic models, will direct the studies on phytoestrogen fate with P. Novak. We have contacted personnel at the Mankato and Brewster wastewater facilities and have permission to sample their effluent.

B. Project Impact and Long-term Strategy: The proposed research fits into a larger research agenda centered at the University of Minnesota that is focused on the problem of environmental estrogens and endocrine disruptors in the State's surface waters. Although the proposed research will be completed in the allotted 3-year period with the requested financial resources, it complements current and prior research in this area. When taken together, the research performed or proposed by the University of Minnesota and its partners (e.g., St. Cloud State University) will provide a more

complete picture of important sources and loads of estrogens/endocrine disruptors, the fate of these compounds in both engineered and natural systems, and potential strategies (communication or engineering) to mitigate the threat caused by these compounds.

C. Other Funds Proposed to be Spent during the Project Period: The three PIs will each devote effort to the project that will be unpaid, 2% for both Novak and Arnold and 3.5% for Schoenfuss.

D. Spending History: None.

VII. DISSEMINATION:

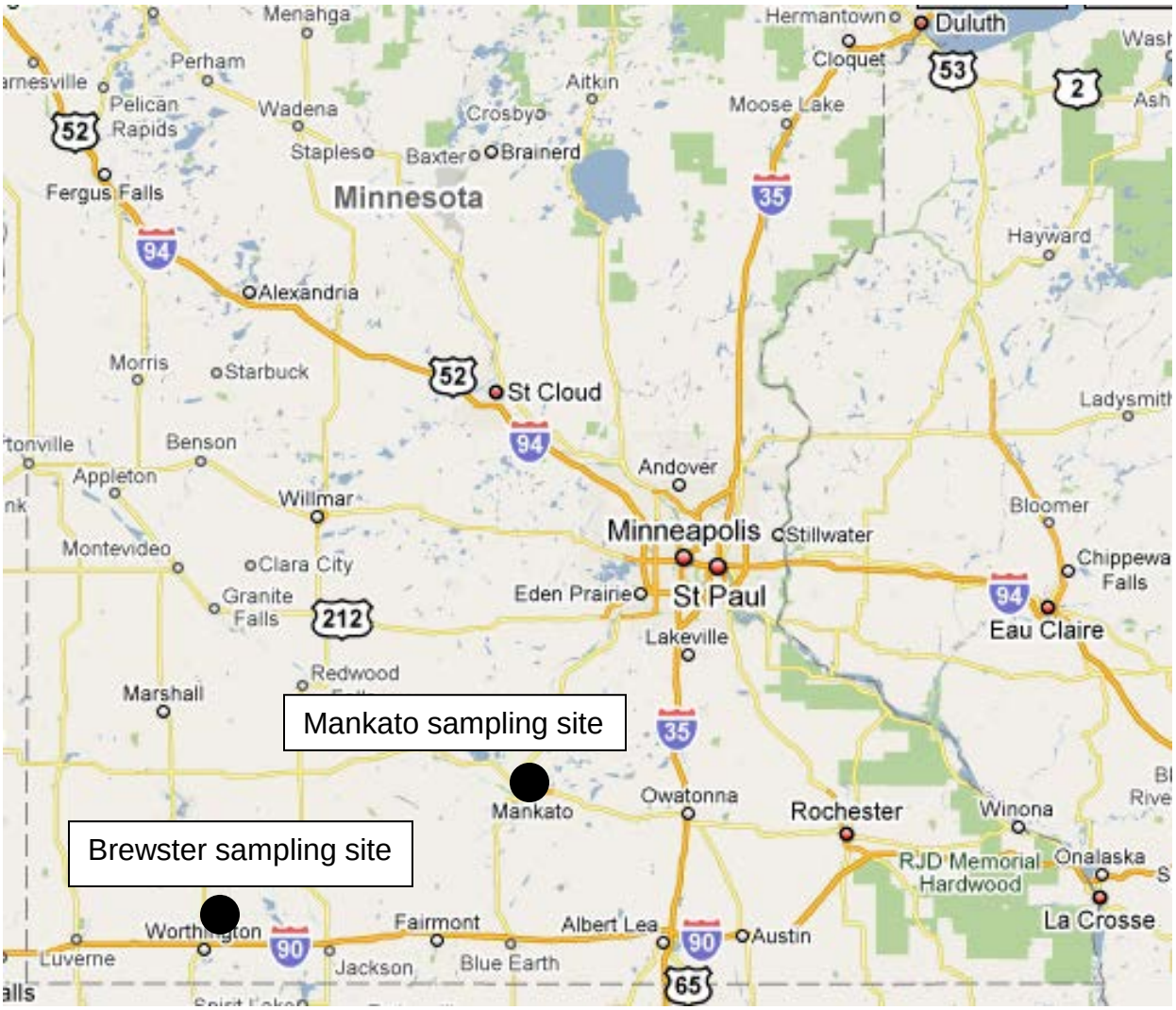
The target audience for results from this research will be professionals in the area of wastewater treatment, watershed management, and industry. Specific targets will be environmental engineers and scientists in academia, industry, state agencies such as the MDA and MPCA, and environmental consultants. Results will be disseminated through scholarly publications in peer-reviewed journals such as *Environmental Science and Technology*. Results from the research project will also be presented at regional conferences such as the *Minnesota Water* conference.

VIII. REPORTING REQUIREMENTS:

Periodic Work Program progress reports will be submitted not later than January 2011, July 2011, January 2012, July 2012, and January 2013. A final Work Program report and associated products will be submitted between June 30 and August 1, 2013 as requested by the LCCMR.

IX. RESEARCH PROJECTS:

See Attachment B.


Menahga
Hermantown
Duluth
Perham
Cloquet
Aitkin
Pelican Rapids
Wadena
Crosby
Moose Lake
Staples
Baxter
Brainerd
Minnesota
Fergus Falls
Hayward
Alexandria
Morris
Starbuck
St Cloud
Benson
Appleton
Andover
Willmar
Bloomer
Minneapolis
Stillwater
Montevideo
Clara City
Chippewa Falls
Granite Falls
Eden Prairie
St Paul
Eau Claire
Lakeville
Redwood
Marshall
Mankato sampling site
Mankato
Owatonna
Rochester
Winona
Brewster sampling site
RJD Memorial Hardwood
Onalaska
La Crosse
Worthington
Fairmont
Albert Lea
Austin
Luverne
Jackson
Blue Earth
52
94
35
53
2
212
90
65

Final Attachment A: Budget Detail for 2010 Projects

Project Title: Fate and ecological impacts of phytoestrogens

Project Manager Name: Paige Novak

Trust Fund Appropriation: $ *340,000*

2010 Trust Fund Budget	**Result 1 Budget:**	**Final Adjusted Result 1 Budget 8/21/2013**	**Amount Spent 06/30/2013**	**Balance 06/30/2013**	**Result 2 Budget:**	**Final Adjusted Result 2 Budget 8/21/2013**	**Amount Spent 06/13/2013**	**Balance 06/30/2013**	**TOTAL BUDGET**	**TOTAL BALANCE**
	Determine the chemical and biological fate of phytoestrogens in surface waters				Determine the impact of the phytoestrogens on fathead minnows					
BUDGET ITEM										
PERSONNEL: wages and benefits	149,000	149,000	149,000	0	~~38,000~~	40,058	40,058	0	189,058	0
Paige Novak (4%)										
William Arnold (4%)										
Graduate Research Assistant (37.5%)										
Graduate Research Assistant (37.5%)										
Subcontract (Some of the work will be conducted at St. Cloud State University (Result 2). The subcontract amount will include Co-PI salary (Heiko Schoenfuss, paid for 2.5% effort), one undergraduate research assistant ($12,000 for 3 years), supplies for experiments (fish, etc., $36,000), and half of the funds required for travel to the site for sampling and in-stream experiments ($1,000)). Graduate research assistants hired and paid through UMN will work on both Result 1 and Result 2 and an additional graduate research assistant will be hired through					82,000	82,000	82,000	0	82,000	0
Other direct operating costs										
Supplies (Laboratory supplies including, but not limited to, gas supply for the LC/MS, glassware, syringes, chemical standards, membrane filters, clean-up columns, and disposables; Analytical costs; Instrument maintenance and repair costs)	~~70,000~~	67,942	67,345	597					67,942	597
Travel expenses in Minnesota	1,000	1,000	682	318					1,000	318
COLUMN TOTAL	**~~$220,000~~**	**$217,942**	**$217,027**	**$915**	**~~$120,000~~**	**$122,058**	**$122,058**	**$0**	**$340,000**	**$915**